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Expression of the Epstein-Barr Virus BHRF1 Gene, a Homologue of Bcl-2, in Nasopharyngeal Carcinoma Tissue

Mei-Ying Liu,[1,2] Ya-Yi Shih,[1] Long-Yuan Li,[1] Sheng-Ping Chou,[3] Tzung-Shiahn Sheen,[4] Chi-Long Chen,[5] Czau-Siung Yang,[1] and Jen-Yang Chen[1,3]*

[1]Graduate Institute of Microbiology, College of Medicine, National Taiwan University
[2]Department of General Education, National Taipei College of Nursing, Taipei, Taiwan
[3]National Health Research Institute, Taipei, Taiwan
[4]Department of Otolaryngology, College of Medicine, National Taiwan University, Taipei, Taiwan
[5]Department of Pathology, College of Medicine, National Taiwan University, Taipei, Taiwan

Epstein-Barr virus (EBV) infection is associated closely with the pathogenesis of nasopharyngeal carcinoma (NPC). The EBV gene product, BHRF1, has been demonstrated in vitro and is structurally and functionally similar to the oncogene bcl-2, that is able to protect cells from programmed cell death. To determine whether the BHRF1 gene is expressed in vivo, BHRF1 mRNA or protein were sought in tissues from NPC and non-NPC patients. BHRF1 transcripts were specifically detected in the NPC tumours (32 out of 44, 72.7%) rather than the non-NPC tissues (0 out of 25) by reverse transcription, polymerase chain reaction and Southern hybridization. Other EBV genes, such as the lytic gene BZLF1 and latent genes EBNA1 and LMP2A, were also investigated. BZLF1 transcripts also were found specifically in NPC tumours (33 out of 44, 75%). EBNA1 was expressed in 79.5% of NPC, and 28% of non- NPC, tissues and LMP2A was expressed in 70.5% of NPC, and 88% of non-NPC, tissues. BHRF1 protein was detected by immunohistochemistry in 4 metastatic NPC, of 36 NPC tissue sections available. The BHRF1 protein was distributed in both the nucleus and cytoplasm of the neoplastic epithelial cells. IgG antibody against the BHRF1 protein was detected in 6 of 17 (35.3%) NPC plasma, but the protein and IgG were both absent from the non-NPC controls. BHRF1 DNA sequences were determined for 11 NPC and 3 non-NPC samples. No sequence was specific for the EBV isolates from NPC tissue. Amino acids 79 and 88 always appeared in the same form, however, for every tested isolate and both were valine or leucine. This particular characteristic was not present in the B95-8 strain or in the corresponding regions of homologues, Bcl-2 and Bcl-XL, and was regarded as unique to Oriental EBV strains. ***J. Med. Virol. 61:241–250, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: nasopharyngeal carcinoma; Epstein-Barr virus; BHRF1 expression; Bcl

INTRODUCTION

Epstein-Barr virus (EBV) infection is believed to be a risk factor for nasopharyngeal carcinoma (NPC), based on serological and molecular studies. Specific antibodies against EBV were shown to be elevated in NPC patients [Henle et al., 1970; Henle and Henle, 1976] even before the onset of the disease [Chen et al., 1985, 1989; Liu et al., 1997]. EBV DNA was found to be present in tumour biopsies from NPC patients [zur Hausen et al., 1970; Wolf et al., 1973]. Viral gene products, mRNAs or proteins, have also been detected in NPC tissues [Fahraeus et al., 1988; Young et al., 1988; Wu et al., 1991; Brooks et al., 1992; Busson et al., 1992; Niedobitek et al., 1992; Chen et al., 1995]. Studies of viral gene expression in NPC have focussed predominantly on latent genes, such as those encoding the latent membrane protein (LMP) and EBV nuclear antigens (EBNA). Some of these latent genes were reported to be associated with the transforming ability of EBV [Cohen et al., 1989; Kaye et al., 1993; Tomkinson et al., 1993]. The expression of a few lytic genes was also investigated in NPC tissues and the results suggested that

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*Correspondence to: Jen-Yang Chen, Graduate Institute of Microbiology, College of Medicine, National Taiwan University, No. 1, Sec. 1, Jen-Ai Rd., Taipei 10018, Taiwan, Republic of China.

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EBV underwent abortive lytic infection in NPC biopsies [Cochet et al., 1993; Sbih-Lammali et al., 1996].

BHRF1, an early EBV gene, encodes a 17 kDa component of the restricted early antigen complex, that is actively expressed in the virus lytic cycle [Pearson et al., 1987]. The BHRF1 protein is structurally and functionally homologous to the oncoprotein Bcl-2 [Cleary et al., 1986] that protects cells from apoptosis [Vaux et al., 1988]. Accumulated evidence has demonstrated that the BHRF1 protein prohibits epithelial cells, including NPC cell lines, from entering apoptosis in vitro [Dawson et al., 1995; Huang et al., 1997; Kawanishi, 1997]. This evidence supports the hypothesis that expression of BHRF1 may be associated with the pathogenesis of NPC [Horner et al., 1995]. Furthermore, the basis of recurrence in treated NPC patients is still not well understood. Based on its ability to prevent apoptosis, expression of the BHRF1 gene has assumed to help neoplastic cells survive treatment. To test this hypothesis, it is important to determine whether BHRF1 is expressed in NPC tissues.

To elucidate the role of BHRF1 in the pathogenesis of NPC, biopsies from primary and metastatic tumours were examined for the presence of BHRF1 transcripts. Latent genes, EBNA1 and LMP2A, and a lytic gene, BZLF1, that have been reported to be expressed in NPC biopsies [Brooks et al., 1992; Cochet et al., 1993; Chen et al., 1995], were also investigated for comparison. In addition, detection of BHRF1 protein in tissues and anti- BHRF1 antibody in plasma enabled the antigen-antibody status of the patients to be determined. Finally, BHRF1 DNA sequences from NPC tissues were analyzed to see if there are any features of the viral strains associated with NPC.

MATERIALS AND METHODS

Biopsies and Plasma Samples

Biopsies were collected from patients attending the National Taiwan University Hospital. Fifty-two biopsies were taken from the nasopharynx of individuals who felt discomfort and were suspected to have NPC. After pathological examination, 27 were confirmed as primary NPC. Of the other 25 cases, 20 were affected by lymphoid hyperplasia; 2, chronic infection; 2, adenoiditis and one, B cell lymphoma. These 25 specimens from non-NPC patients were used as controls. Furthermore, seventeen biopsies, including tissue from lymph nodes, liver, skin, lung or soft tissue, were taken from NPC patients with metastases. All the samples were examined by histopathology. Some plasma samples were available and these were stored at –20°C for later detection of antibody.

Cell Lines

EBV-positive lymphoblastoid cell lines, including Raji [Pulvertaft, 1965], B95-8 [Miller et al., 1972], P3HR1 [Hinuma and Grace, 1967] and Akata [Takada et al., 1991], and the EBV-negative lymphoblastoid cell line BJAB [Menezes et al., 1975] were grown in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum. Induction of EBV replication was carried out by superinfection of Raji cells, treatment with 12-*o*-tetradecanoylphorbol-13-acetate and sodium butyrate for B95-8 or P3HR1 and treatment with 0.5% anti-human IgG for Akata cells.

Isolation of RNA and DNA

Tissue samples were frozen in liquid nitrogen or stored at –80°C before use. To extract nucleic acid and protein from the biopsies, each 50–100 mg of tissue was homogenized and lysed in 1 ml TRIzol reagent (GIBCO, BRL, CA) using a Dounce homogenizer. Cultured cells were resuspended and lysed in the same reagent at a concentration of $5–10 \times 10^6$ cells/ml. After 5 min incubation at room temperature, chloroform was added for extraction. After centrifugation, RNA remained in the upper aqueous phase and DNA partitioned to the lower organic phase. The RNA fraction was isolated and precipitated with isopropanol. The RNA was pelleted by centrifugation and washed with 75% ethanol. Finally, the pellet was resuspended in RNase-free distilled water. The DNA fraction was precipitated with absolute alcohol, according to the manufacturer's instructions. The resulting DNA precipitates were then resuspended in 10 mM Tris-HCl, pH8.0, and 1 mM EDTA.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To synthesize the first strand of complementary DNA in an RT reaction, 1 μg RNA was annealed with hexanucleotide random primers (Boehringer Mannheim, Germany) and then deoxynucleotides were incorporated with 200 units of SUPERSCRIPT™ RNase H free reverse transcriptase (GIBCO BRL, CA) at 42°C for 50 min. At completion, the enzyme was inactivated by heating at 70°C for 15 min. The newly synthesized cDNA was then amplified by PCR using DyNAzyme™ II DNA polymerase (FINNZYMES OY, Finland). Primers located in exons of each gene were used to differentiate RNA from possible DNA contamination. Primer sequences for each PCR are listed in Table I. One cycle of DNA synthesis was initiated by denaturation at 94°C for 30 sec, followed by annealing with primers at specific temperature for 1 min and DNA extension at 72°C for 2 min. The above procedures were run for 32 cycles for each amplification. Annealing temperatures for each primer pair were as follows: 42°C for primers BHRF1-1 and BHRF1-2 to amplify the BHRF1 gene; 55°C for primers BZLF1-1 and BZLF1-2 to amplify the BZLF1 gene; 58°C for primers EBNA1-1 and EBNA1-2 to amplify the EBNA1 gene and 45°C for primers LMP2A-1 and LMP2A-2 to amplify the LMP2A gene. Hypoxanthine phosphoryltransferase (HP), a house keeping gene, was amplified as an internal control for RNA quality using primers HP-1 and HP-2 and an annealing temperature of 50°C. Detection of EBER is the most sensitive method for detection of EBV because it is the most abundant viral transcript in EBV-infected cells [Howe and Shu, 1989]. Therefore, primers EBER-1

TABLE I. Sequences of Oligonucleotides Used in This Study

Primers	Sequence (5′-3′)	Coordinates in B95-8	Reference
HP-1	TATGGACAGGACTGAACGTC	—	Martel-Renoir et al., 1995
HP-2	GTTGAGAGATCATCTCCAACC	—	Martel-Renoir et al., 1995
BHRF1-1	CTGGAGATAAATAAATAAC	54,938–54,920	—[a]
BHRF1-2	GTCAAGGTTTCGTCTGTGTG	53,830–53,849	Sam et al., 1993
BHRF1-P	ATGGCCTATTCAACAAGGGA	54,376–54,395	—[a]
BZLF1-1	ACATCTGCTTCAACAGGAGG	102,284–102,303	Cochet et al., 1993
BZLF1-2	TTACACCTGACCCATACCAG	103,118–103,099	Cochet et al., 1993
EBNA1-1	TTAGGAAGCGTTTCTTGAGC	67,483–67,502	Tierney et al., 1994
EBNA1-2	GATCGGATCCTGTTCCACCGTGGGT	108,216–108,192	—[a]
EBNA1-P	AGAGAGTAGTCTCAGGGCAT	67,544–67,563	Brooks et al., 1992
LMP2A-1	ATGACTCATCTCAACACATA	166,874–166,893	Brooks et al., 1992
LMP2A-2	CATGTTAGGCAAATTGCAAA	380–361	Brooks et al., 1992
LMP2A-P	ATCCAGTATGCCTGCCTGTA	62–81	Brooks et al., 1992
EBER-1	CTACGCTGCCCTAGAGGTTTTGCTA	6,658–6,682	Takeuchi et al., 1996
EBER-2	ATGCGGAGCACCAGCTGGTACTTGA	6,814–6,790	Takeuchi et al., 1996
BHRF1-3	TAAAGTGCTCGAGAAAATGT	54,976–54,957	—[a]
BHRF1-4	GGAATTCGTCGACCAGATCTTGTTGAGCAA	54,358–54,375	—[a]
BHRF1-5	TTGGGTCTCCACGGTGAAAT	54,664–54,645	—[a]
BHRF1-6	ATTTCACCGTGGAGACCCAA	54,645–54,664	—[a]
T7	TAATACGACTCACTATAGGGCGA	—	—[b]
SP6	ATTTAGGTGACACTATAGAATAC	—	—[b]

[a]Designed by this laboratory.
[b]T7 and SP6 were sequences flanking the insert of plasmid pGEM-T.

and EBER-2 were used to amplify EBER cDNA, using an annealing temperature of 60 °C.

Southern Hybridization

Specificity of the PCR products were confirmed by Southern hybridization [Southern, 1975] using internal oligonucleotides as probes. The probes were prepared by end labeling with [γ-^{32}P]dATP (Amersham, England) using T4 polynucleotide kinase (New England Biolabs, England). The hybridization temperature for probes BHRF1-P, EBNA1-P and LMP2A-P was 50°C. After hybridization for 18 to 20 hr, the membranes were washed with 900 mM NaCl, 6 mM EDTA, 90 mM Tris-HCl, pH 8.0, and 0.5% SDS at 25°C for 10 min, four times and then with the same buffer at 50°C for 1 min. Finally, the membranes were washed again in the same buffer at 25°C for 10 min and then subjected to autoradiography. The probe used to detect the PCR products of the BZLF1 gene was a 1.6 kb, overlapping DNA fragment labeled with [α-^{32}P]dCTP (Amersham, England) using a Random Primed DNA Labeling Kit (Boehringer Mannheim, Germany). Hybridization was carried out at 65°C and the membrane was washed in 15 mM NaCl, 1.5 mM Tris-sodium citrate, pH7.0, and 0.1% SDS at 65°C for 3 hr with 3 changes of the buffer.

Immunohistochemistry

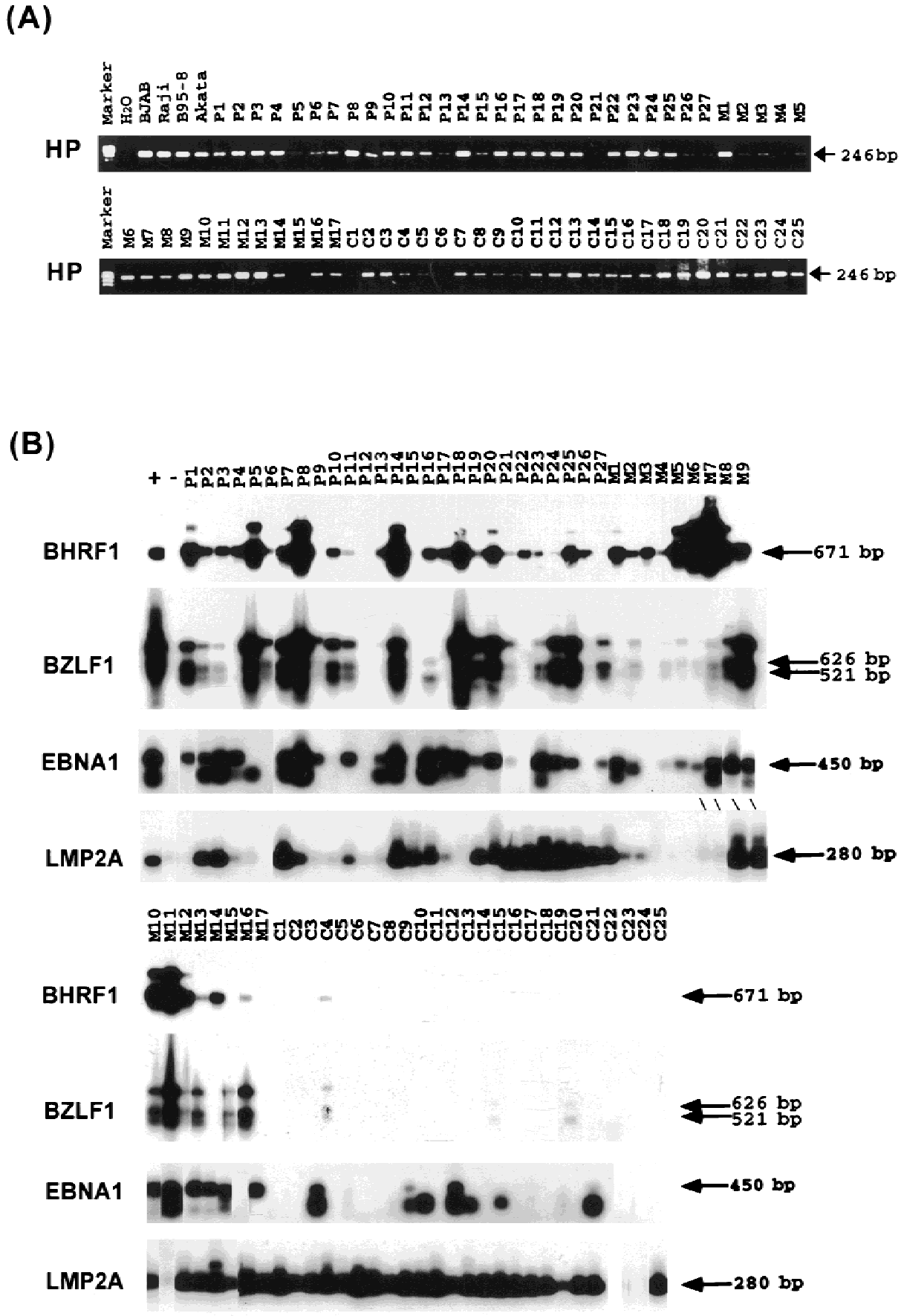
Sections of paraffin-embedded biopsies were tested for the BHRF1 protein by immunostaining with a mouse monoclonal antibody, mAb 5B11 (ABi Inc., Columbia, MD). Antigens in the paraffin-embedded sections were made more immunoreactive by microwave heating [Swanson, 1994]. Briefly, the sections were deparaffinized, hydrated, and boiled in 10 mM citrate buffer, pH 6, for 5 to 10 min by microwaving twice. Subsequently, the sections were probed with the anti-BHRF1 antibody, 5B11, as the primary antibody. To enhance the sensitivity of the specific reaction, biotin-conjugated anti-mouse IgG was added as the secondary antibody, followed by streptavidin-conjugated horseradish peroxidase (HRP) [Hsu and Raine, 1984]. The antigen reactivity was detected by incubating the sections with 3,3′-diaminobenzidine tetrahydrochloride (Dako, CA) that is the substrate for HRP and the background was counterstained by hematoxylin.

Western Blot Analysis of Anti-BHRF1 Antibody in Plasmas

To detect the presence of antibody against the BHRF1 protein, the plasmas available from some of the biopsy donors were examined by western blot analysis. Briefly, recombinant BHRF1 protein expressed in bacteria was used as the antigen as described previously [Liu et al., 1998]. Human plasmas were diluted by 200-fold and reacted with the BHRF1 protein-bound membrane. HRP-conjugated anti-human IgG antibody (CAPPEL, Organon Teknika Corp., NC) was used as the secondary antibody. The 5B11 mAb and serum from a child who was no infected with EBV were used as the positive and negative controls, respectively. The blots were developed using an ECL western blotting detection system (Amersham, Buckinghamshire, UK) and then exposed to X-ray film. The results were quantified by scanning the film with program FREEMAX V3.0 (Media Cybernetics, L.P., MD) and the values obtained classified as described previously.

Cloning and Sequencing of the BHRF1 Gene from Biopsies

DNA samples from eleven patients, of whom 6 had primary NPC and 5, metastatic NPC, and from three


(A)
Marker H2O BJAB Raji B95-8 Akata P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14 P15 P16 P17 P18 P19 P20 P21 P22 P23 P24 P25 P26 P27 M1 M2 M3 M4 M5
HP
246bp
Marker M6 M7 M8 M9 M10 M11 M12 M13 M14 M15 M16 M17 C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 C14 C15 C16 C17 C18 C19 C20 C21 C22 C23 C24 C25
HP
246 bp
(B)
+ - P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14 P15 P16 P17 P18 P19 P20 P21 P22 P23 P24 P25 P26 P27 M1 M2 M3 M4 M5 M6 M7 M8 M9
BHRF1
671 bp
BZLF1
626 bp
521 bp
EBNA1
450 bp
LMP2A
280 bp
M10 M11 M12 M13 M14 M15 M16 M17 C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 C14 C15 C16 C17 C18 C19 C20 C21 C22 C23 C24 C25
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EBNA1
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LMP2A
280 bp

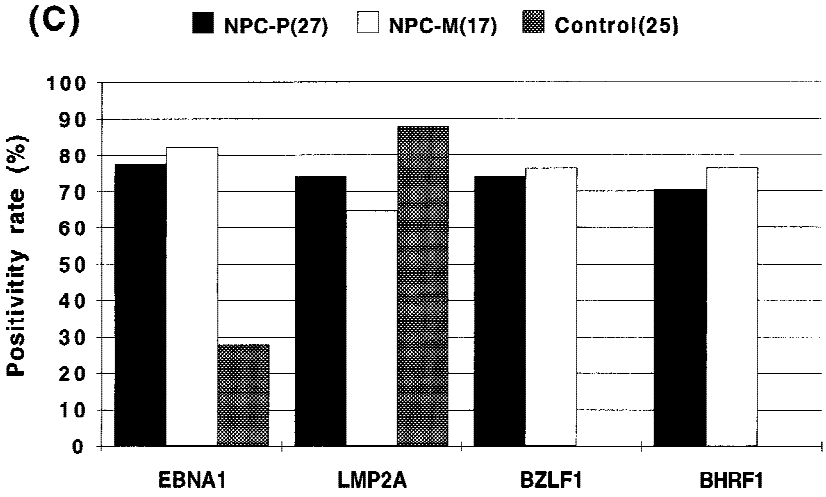

Fig. 1. Detection of EBV lytic and latent gene expressions by RT-PCR and Southern hybridization. RNA was extracted from biopsies from primary NPC (P), metastatic NPC (M) and non-NPC controls (C), converted to cDNA by reverse transcription, and amplified for a house keeping gene, HP, by PCR (panel A). Equal amounts of the cDNA were amplified for BHRF1, BZLF1, EBNA1 and LMP2A, respectively, using specific primers and the PCR products were confirmed with internal probes by Southern hybridization (panel B). The expected sizes of each gene product are indicated by arrows. +, EBV positive cell lines as the positive controls: Akata cells for BHRF1; P3HR1 cells for BZLF1, EBNA1 and LMP2A. –, EBV negative cell line BJAB as the negative control. Positivity rates for each transcript for the various patients are plotted in panel C.

non-NPC controls were selected randomly. The BHRF1 DNA fragment was amplified by the PCR using primers BHRF1-3 and BHRF1-4 (Table I). PCR products from three independent reactions were pooled and cloned in the vector pGEM-T (Promega, Madison, WI). The recombinant plasmids were introduced into *Escherichia coli* by transformation. Plasmids from two transformants were prepared separately, and the inserts were sequenced [Sanger et al., 1977] using an ABI PRISMTM Dye Terminator Cycle Sequencing Core Kit (Perkin-Elmer, CA). Because the length of the BHRF1 ORF is 573 bp, which is too long for a sequencing run, four primers were used for the sequencing reactions, including primers T7 and SP6 located in the flanking sequences of the vector and primers BHRF1-5 and BHRF1-6 located in the middle of the ORF (Table I). Sequences of double stranded DNA were read automatically by a 373A DNA sequencer (Applied Biosystems, Inc., CA). If the results from the two transformants were different, another clone was selected for analysis to confirm the nucleotide sequence.

RESULTS
Detection of BHRF1 Transcripts in NPC Tumours

To investigate whether the BHRF1 gene is expressed in NPC tumours, RNA was extracted from biopsies from patients with NPC and non-NPC diseases. The quality of the RNA was confirmed by detecting the HP house keeping transcript using an RT-PCR based method, as are shown in Figure 1A. Most of the samples were of good quality and a 246 bp fragment was amplified. A few samples, however, were shown to contain very low amounts of intact RNA. Equal amounts of the cDNA products were amplified by different pairs of primers, designed to detect mature RNA transcripts of specific EBV genes, including BHRF1, BZLF1, EBNA1 and LMP2A. The resulting PCR products were detected by Southern hybridization using, as probes, a DNA fragment or synthetic oligonucleotides complementary to a sequence located in the exon of each gene and internal to each amplified DNA fragment (Fig. 1B). For the BHRF1 transcript, 19 of 27 primary NPC biopsies and 13 of 17 metastatic NPC biopsies clearly gave positive signals. BHRF1 mRNA was not detected in any of the non-NPC controls. Bands indicated by arrows are 671 bp products derived from spliced BHRF1 mRNA, those with a low intensity, such as in case C4, were too faint to be considered unequivocally positive. The larger bands were products derived from unspliced mRNA or residual genomic DNA contaminating the RNA preparations. Similarly, expression of the BZLF1 gene was demonstrated in 20 primary NPC biopsies, 13 metastatic NPC biopsies and none of the non-NPC controls. Two products, a 626 bp and a 521 bp, were derived from differentially spliced RNAs of the BZLF1 gene. For the EBV latent genes, EBNA1 transcripts were detected by amplifying a 450 bp product in 21 primary NPC biopsies, 14 metastatic NPC biopsies and 7 non-NPC controls. LMP2A mRNA was detected by amplifying a 280 bp DNA fragment in 20 primary NPC biopsies, 11 metastatic NPC biopsies and 22 non-NPC controls. Four of the biopsies, P12 and C22-24, were negative for all four transcripts. The absence of EBV was excluded because EBER1, which is one of the most abundant transcripts in EBV latently infected cells, could be detected in the RNA preparations using RT-PCR. Positivity rates of detection of the two lytic and the two latent transcripts are plotted in Figure 1C. A clear observation is that none of the non-NPC controls expressed either of the lytic genes, BHRF1 or BZLF1. Many biopsies from NPC and non-NPC samples showed expression of LMP2A. EBNA1 was expressed in NPC biopsies more frequently than in the non-NPC controls. Another important finding is that the BHRF1 gene, that has been shown in vitro to have anti-apoptosis properties, was expressed in most NPC biopsies but not in the non-NPC controls. The detection frequencies for the various transcripts were similar for primary and metastatic NPC biopsies, indicating that there were no detectable differences in ex-

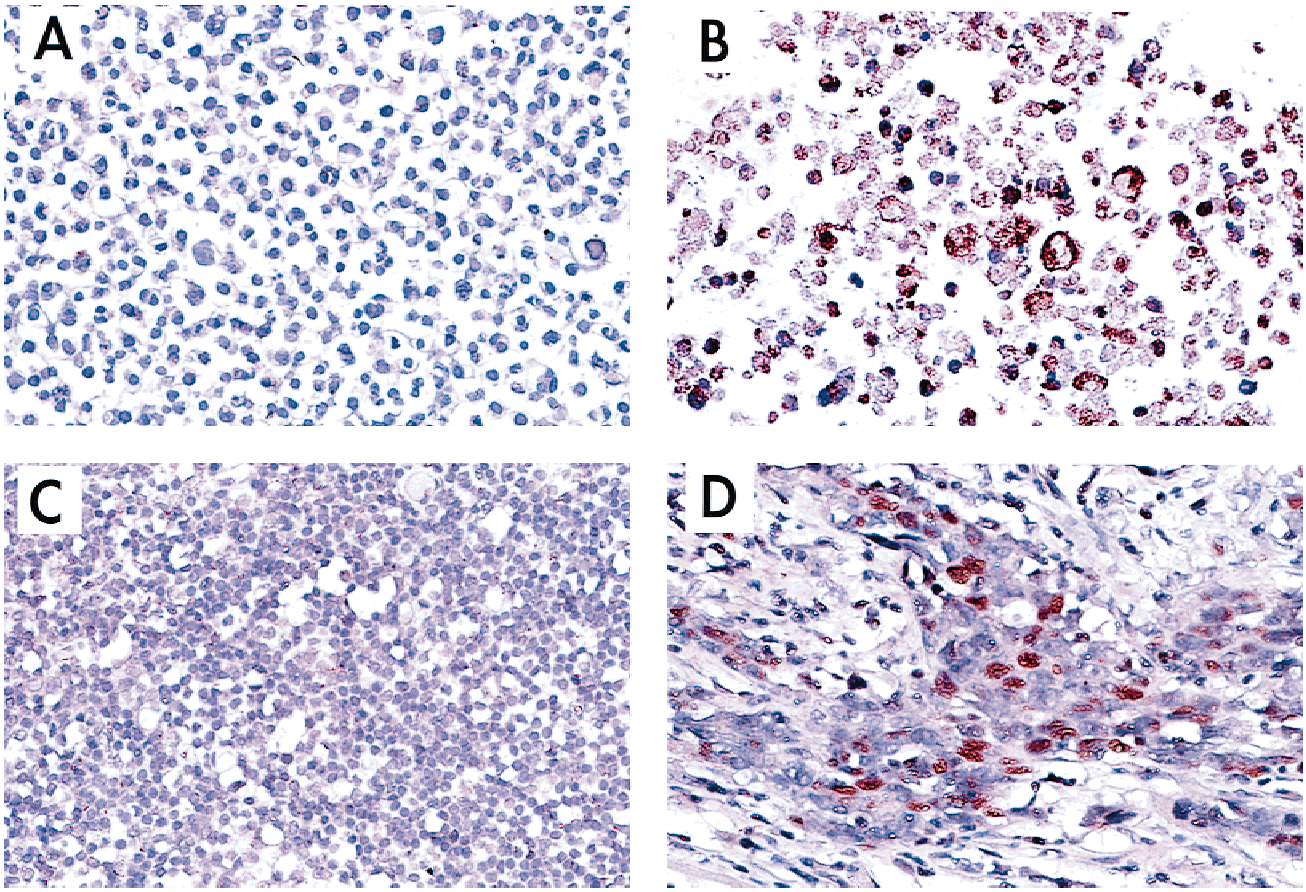

Fig. 2. BHRF1 protein expressed in NPC neoplastic cells. Uninduced P3HR1 cells (A), P3HR1 cells induced by 12-*o*-tetradecanoylphorbol-13-acetate and sodium butyrate (B), non-NPC control C2 (C) and metastatic NPC M2 (D) were probed with 5B11 monoclonal antibody and immunohistochemistry staining.

pression of these EBV genes, even when the tumours had spread.

Expression of BHRF1 Protein in NPC Tissue Sections

Expression of the BHRF1 protein in the biopsies was detected by immunohistochemistry using monoclonal antibody 5B11 as the probe. Of the 25 non-NPC controls, tissue sections were available for 20, and all gave negative results. Of the 27 primary NPC biopsies, 21 paraffin-embedded tissue sections were available and were stained with the specific antibody. The BHRF1 protein, however, could not be detected in any of them. Of the 17 metastatic NPC biopsies, 15 were examined and 4 clearly showed positive cells within the malignant regions (26.7% positive). Representative results are shown in Figure 2. P3HR1 is an EBV positive B lymphoblastoid cell line and BHRF1 protein could barely be detected before induction (Fig. 2A). In contrast, BHRF1 was expressed at high levels in the cytoplasm after induction (Fig. 2B). The protein is absent from the C2 non-NPC biopsy (Fig. 2C) but present in the M2, metastatic NPC biopsy (Fig. 2D). Interestingly, the BHRF1 protein in vivo seemed to localize in both the nucleus and cytoplasm of the epithelial cells. Three other metastatic NPC cases showed a similar pattern of staining.

Presence of Anti-BHRF1 Antibody in NPC Plasmas

It has been reported frequently that antibodies against EBV proteins are elevated in NPC patients' sera. Because the BHRF1 transcript could be detected in most NPC patients, it was interesting to look at their immune responses to the BHRF1 protein. Unfortunately, only 12, 5 and 6 plasma samples were available for the groups of primary NPC, metastatic NPC and non-NPC controls, respectively. The samples were tested for specific IgG antibody to the BHRF1 protein by western blotting, using recombinant BHRF1 antigen. The results are shown in Figure 3. BHRF1 antibody positivity rates were 33.3% (4/12) for primary NPC, 40% (2/5) for metastatic NPC and 0% (0/6) for the non-NPC controls. To co-relate the presence of BHRF1 antibody with the data for other EBV products, the results are summarized in Table II. Of the 23 cases for whom plasma was available, most NPC biopsies, but

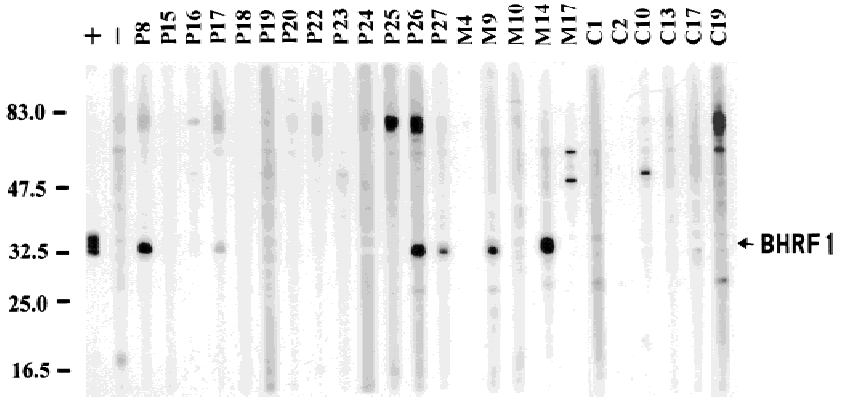

Fig. 3. Western blot analysis of IgG antibody against recombinant EBV BHRF1 protein. *E. coli* extracts containing recombinant BHRF1 protein were used as antigens to react with plasma from NPC and non-NPC patients. +, 5B11 monoclonal antibody as a positive control; −, an EBV uninfected human serum as a negative control. Molecular sizes are shown in kDa.

TABLE II. EBV Status in Individuals*

Patient number	WHO stage	EBER1	BHRF1 mRNA	BHRF1 protein	BHRF1 IgG	EA IgA	VCA IgA
P8	III	+	+	−	++	1:160	1:160
P15	II	+	−	−	−	1:10	1:10
P16	III	+	+	−	−	1:80	1:160
P17	III	+	+	−	+	1:40	1:160
P18	III	+	+	−	−	−	1:40
P19	III	+	(+)	−	−	1:10	−
P20	III	+	+	−	−	1:10	1:40
P22	III	+	+	−	−	1:10	1:10
P23	III	+	+	−	−	1:40	1:40
P24	III	+	(+)	−	−	1:40	1:80
P25	III	+	+	−	−	1:20	1:40
P26	III	+	+	−	++	1:40	1:80
P27	III	+	−	−	+	ND	1:160
M4	II	+	(+)	−	−	1:10	1:10
M9	III	+	+	(+)	++	1:320	1:640
M10	III	+	+	−	−	1:160	1:160
M14	III	+	+	−	++	1:320	1:640
M17	I	+	−	−	−	−	1:10
C1	−	+	−	−	−	−	1:10
C2	−	+	−	−	−	−	−
C10	−	+	−	−	−	−	−
C13	−	+	−	−	−	−	1:10
C17	−	+	−	−	−	−	−

*WHO stage of NPC and EA IgA and VCA IgA antibody status were kindly provided by clinical colleagues. P, patients with primary NPC; M, patients with metastatic NPC; C, patients with non-NPC diseases of the nasopharynx as controls; ND, not done; +, positive; −, negative; (+), weak reaction below the positive/negative cut off.

none of the non-NPC controls had detectable BHRF1 transcripts. Of those mRNA positive individuals, only M9 had a very weak signal for the BHRF1 protein and was considered negative. This negativity result, however, is very possibly due to the low sensitivity of the detection method. There were 6 cases showing clearly antibody reactivity, indicating that the BHRF1 protein must have been expressed during the course of NPC development. Case P27 was positive for BHRF1 antibody but tested negative for both the BHRF1 mRNA and protein. Instability of the RNA and protein may be the cause of this disparity or, perhaps, antibody relates to previous expression of the gene. Two other antibodies, IgA to EBV EA and VCA, are generally accepted as markers for detection of NPC, and were present in most of the NPC patients. These showed good correlation, with some exceptions (Table II).

Comparison of BHRF1 cDNA Sequences of Different Origin

The DNA sequences of EBV BHRF1 from 14 patients, who were suffering from primary NPC, metastatic NPC or other diseases with symptoms mimicking NPC, were analysed. In addition, EBV BHRF1 sequences were determined for the B95-8 and Akata cell lines maintained in this laboratory. Compared to the B95-8 DNA sequence, several nucleotide substitutions were detected but none was found consistently among the NPC patients (data not shown). Some amino acid substitutions were resulted from these nucleotide changes (Table III). The most frequent substitution was from valine to leucine at amino acid 79 and was commonly observed in the NPC patients (8 out of 11) but not in the three non-NPC controls. Conversely,

TABLE III. Variation of Amino Acids in the BHFR1 Protein Derived From Different EBV Isolates and Comparison With Its Homologues Bcl-2 and Bcl-XL

Sample	Geographical origin	Variant amino acid positions									
		7	24	38	41	74	79	80	88	112	184
B95-8	American LCL	Glu	Thr	Leu	Ser	Thr	Val	Asp	Leu	Cys	Leu
Akata	Japan BL cell line	–	–	–	–	–	–	–	Val	–	–
P2	Taiwan nasopharynx	–	–	–	+	–	Leu	+	–	+	–
P3	Taiwan nasopharynx	–	–	–	+	–	Leu	+	–	+	–
P5	Taiwan nasopharynx	–	–	–	–	–	Leu	–	–	–	–
P8	Taiwan nasopharynx	–	–	–	–	–	–	–	Val	–	–
P14	Taiwan nasopharynx	Asp	Ala	Pro	–	–	Leu	Glu	–	–	–
P23	Taiwan nasopharynx	–	–	–	+	–	Leu	+	–	+	+
M1	Taiwan soft tissue	–	–	–	–	–	–	+	Val	–	–
M7	Taiwan lymph node	–	–	–	–	Arg	Leu	Asp	–	–	–
M11	Taiwan lymph node	–	–	–	+	–	Leu	+	–	+	–
M12	Taiwan soft tissue	–	–	–	–	–	–	+	Val	–	–
M13	Taiwan soft tissue	Asp	Ala	Pro	–	–	Leu	+	–	+	–
C4	Taiwan lymphoid hyperplasia	–	–	–	–	–	–	–	Val	–	–
C8	Taiwan adenoid	–	–	–	–	–	–	–	Val	–	–
C21	Taiwan lymphoid hyperplasia	–	–	–	–	–	–	–	Val	–	–
Bcl-2	(Cleary et al., 1986)	–	Ala	Pro	–	Leu	Ala	Arg	Lys	–	–
Bcl-X	(Boise et al., 1993)	–	Gln	Arg	Thr	–	Ala	Tyr	Lys	–	–

LCL, lymphoblastoid cell line; BL, Burkitt's lymphoma; –, the identical amino acid with the same triplet codon as in B95-8 BHRF1; +, the same amino acid as in B95-8 BHRF1 but with a change in the codon.

amino acid 88 was changed from leucine to valine in all the biopsy samples that did not show the substitution of leucine for valine at amino acid 79. The properties of valine and leucine, however, are very similar: both are neutral and hydrophobic and, therefore, not many effects are expected from these substitutions. An interesting conclusion can be drawn that the Oriental strains of EBV, regardless of Burkitt's lymphoma, NPC or non-malignant origin, encode the same amino acids at positions 79 and 88, either leucine or valine. The symmetry may be important for protein structure. NPC biopsies N14 and M13 had substitutions of aspartic acid for glutamine at amino acid 7, alanine for threonine at amino acid 24 and proline for leucine at amino acid 38. Only 2 of the 11 NPC biopsies showed this pattern. In addition, further amino acid substitutions were detected in NPC patient M7. The consequence of these changes remains to be determined. After alignment analysis, all the transitions described above were found outside the consensus regions of the Bcl-2 family (Table III). This polymorphism may not affect the function of the BHRF1 protein; however, possible effects on the protein properties, such as stability, cannot be excluded.

DISCUSSION

BHRF1 is a member of the bcl-2 family and has been demonstrated to protect cells from apoptosis in different settings in vitro. The role of BHRF1 in vivo has not yet been determined. The gene has been reported to be transcribed in EBV-associated B-cell lymphomas [Oudejans et al., 1995] and a nasal NK/T cell lymphoma [Chiang et al., 1996]. Our study is the first to demonstrate that BHRF1 is expressed in NPC tissues. BHRF1 mRNA and protein were detected in the NPC biopsies. Most BHRF1 mRNA positive NPC cases, however, were negative for the BHRF1 protein. The low detection rate of the protein might be improved by using an antibody probe with higher affinity. If the cause was due to low stability of the BHRF1 protein, the procedures involved in processing tissue samples for immunohistochemistry may need to be modified. Another possibility that cannot be excluded is that the detected BHRF1 transcripts might be derived from infiltrating lymphocytes. It would be very likely that microenvironment differed in the NPC samples compared to the normal lymphoepithelium and resulted in the differences in EB viral gene expression in these infiltrating cells. After all, unlike EBNA1 and LMP2A, BHRF1 could hardly be detected in non-malignant nasopharyngeal tissues affected by other diseases, of which most were lymphoid hyperplasia.

In a limited number of the immunohistochemistry positive cases, the BHRF1 protein was clearly shown to be located in cancer cells rather than normal cells. Moreover, it was detected in the nuclei as well as cytoplasm, where BHRF1 was found localized in lymphocytes. Coincidentally, similar results were also found in the epithelial cell line SCC12F, in which BHRF1 was over-expressed [Dawson et al., 1998]. Thus, the cellular localization of BHRF1 seems to depend on the cell type.

The expression of other EBV genes was investigated in parallel to BHRF1. The positivity frequencies for the EBNA1 (79.5%) and LMP2A (70.5%) transcripts among the NPC patients were rather lowed than the data reported by other laboratories[Busson et al., 1992; Brooks et al., 1992; Chen et al., 1995]. This discrepancy may be attributable to variability in sensitivity of detection between laboratories. The sizes of the samples in other studies, however, were smaller than here. The majority (88%) of the non-NPC patients we examined showed expression of LMP2A. This is not surprising because most of them were affected by lymphoid hyperplasia and LMP2A had been reported to be the only

EBV gene product detectable in peripheral blood cells [Qu and Rowe, 1992]. Seventy-five percent of the NPC patients examined (33 of 44) were found to express BZLF1, the first gene expressed in the EBV lytic cycle. This result is consistent with a previous finding by Cochet et al. [1993]. In the study, BZLF1 expression was detected in all seven NPC subjects investigated. More interestingly, we found that BZLF1, like BHRF1, was not transcribed in biopsies from the non-NPC group.

Although BHRF1 is functionally and structurally similar to bcl-2, it can be differentiated from bcl-2 in many ways. The BHRF1 protein does not form a heterodimer with Bax [Theodorakis et al., 1996], whereas Bcl-2 can be associated with, and negatively regulated by, Bax [Oltvai et al., 1993; Chittenden et al., 1995]. Recently, it was reported that BHRF1 had more profound effects than Bcl-2 in inhibiting epithelial differentiation and in promoting cell proliferation [Dawson et al., 1998]. As for distribution, Bcl-2 was expressed in both neoplastic and normal epithelial cells of the nasopharynx [Lu et al., 1993]. Our findings for BHRF1 are different from these observations for Bcl-2. BHRF1 mRNA was detected only in NPC tissues and protein expression was restricted to neoplastic epithelial cells. Although BHRF1 and Bcl-2 belong to the same family and both show anti-apoptosis function, there are still many differences between them.

The detection of anti-BHRF1 IgG in NPC patients was relatively infrequent in this study. Approximately 35.3% (6 out of 17) of the NPC patients were positive for the antibody, that was far below the 61.3% (57 out of 93) that we reported in another serological study [Liu et al., 1998]. A small sample size may be one of the reasons for this low frequency. Another possible explanation for the difference may reside in the fact that the samples we assayed were plasma instead of sera. It is not known whether the anti-coagulant present in plasma may interfere with the immunological reaction in western blot analysis. This question can be answered by investigating a collection of paired serum and plasma from NPC patients.

Comparing BHRF1 DNA sequence among different isolates, we did not find any particular pattern associated with NPC, that was consistent with the findings of other laboratories [Khanim et al., 1997]. Nevertheless, Theodorakis et al. [1996] introduced some mutations into BHRF1 expression plasmids and found that several amino acid substitutions could enhance the ability of the BHRF1 protein to promote cell proliferation. Parallel changes did not occur in any sequences we obtained from NPC biopsies. A unique arrangement of amino acids 79 and 88, however, was found in our study. These two amino acids are both valine or leucine, regardless of the virus isolates from NPC or non-NPC biopsies. This phenomenon was seen in both the Akata strain and the Taiwanese strains (Table III) and may be considered as a character of Oriental EBV strains.

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